Veterinary Immunology and Immunopathology 131 (2009) 97–104

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Role of nitric oxide production in dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*

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ARTICLE INFO

Article history:
Received 23 February 2009
Received in revised form 23 February 2009
Accepted 30 March 2009

Keywords:
Mycobacterium avium subsp. *paratuberculosis*
Nitric oxide
Cytokines
Cattle

ABSTRACT

Nitric oxide (NO) is a crucial mediator in host defense and is one of the major killing mechanisms within macrophages. Its induction is highly affected by the types of cytokines and the infectious agents present. In the current study, NO production was evaluated after in vitro infection of unfractionated peripheral blood mononuclear cells (PBMCs) with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) after 8 h, 3 and 6 days of culture for cows in different stages of disease. In addition, the effects of in vitro exposure to inhibitory cytokines such as interleukin-10 (IL-10) and transforming growth factor β (TGF-β) as well as the pro-inflammatory cytokine IFN-γ were correlated with the level of NO production. Nitric oxide production was consistently higher in cell cultures from subclinically infected animals at all time points. An upregulation of NO production was demonstrated in unfractionated cell cultures from healthy control cows after exposure to MAP infection as compared to noninfected cell cultures. A similar increase in NO due to the addition of MAP to cell cultures was also noted for clinically infected cows. NO level among subclinically infected cattle was greater at all time points tested and was further boosted with the combination of both in vitro MAP infection and IFN-γ stimulation. Alternatively, nonspecific stimulation with LPS from *Escherichia coli* O111:B4-W resulted in an upregulation of NO production in all infected groups at 3 and 6 days after in vitro infection. Finally, the in vitro exposure to inhibitory cytokines such as IL-10 and TGF-β prior to MAP infection or LPS stimulation resulted in the downregulation of this inflammatory mediator (NO) in all experimental groups at all time points. In summary, a higher level of NO production was associated with cows in the subclinical stage of MAP infection. As well, the results demonstrated an increase in NO production upon infection with MAP and in the presence of exogenous IFN-γ. Finally, the results suggest an important role of IL-10 and TGF-β on the profile of NO production which may explain the low NO production in MAP clinically infected cows.

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1. Introduction

Johne's disease is a chronic enteric disease characterized by intermittent diarrhea, weight loss and death. The causative agent of Johne's disease is *Mycobacterium avium* subsp. *paratuberculosis* (MAP), an intracellular pathogen that resides and replicates within the phagosome of nonactive macrophages (Hostetter et al., 2002). Activation of macrophages by treatment with recombinant IFN-γ and LPS has resulted in increased acidification and maturation of phagosomes and a decrease in the number of viable MAP within macrophages (Hostetter et al., 2002). Further,

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0165-2427/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.vetimm.2009.03.020

treatment of MAP-infected bovine monocytes with IFN-γ resulted in an increase in NO production by macrophages (Zhao et al., 1997). Nitric oxide induction has been directly related to the capacity of IFN-γ-activated murine macrophages to inhibit or kill *M. tuberculosis*, *M. avium*, and *M. leprae* in vitro (Adams et al., 1991; Chan et al., 1992; Denis, 1991). Although the chemically generated NO in cell-free medium has been observed to kill ingested MAP, the NO released upon activation of bovine monocytes with rIFN-γ has been associated with only modest increases in antimycobacterial activity (Zhao et al., 1997). In addition, at certain concentrations, NO markedly inhibits Th1 cell proliferation, thus serving as a potential co-regulator of the Th1/Th2 balance (MacLean et al., 1998; Taylor-Robinson et al., 1994).

The stages of Johne's disease (i.e., subclinical and clinical) reflect an ongoing struggle with host immunity. The induction of IFN-γ that occurs in the contained (subclinical) stage of disease represents an activation of the host cell-mediated immune response (Sweeney et al., 1998). Animals in the excretory subclinical stage are reported to have high IFN-γ gene expression locally at the site of infection (Sweeney et al., 1998). This response is downregulated when animals progress to the clinical stage of the disease while other pro-inflammatory cytokines such as IL-1β and IL-6 become elevated (Lee et al., 2001; Stabel, 2000). Several reports have correlated the presence of IL-10 and TGF-β with the clinical stage of MAP infection (Karcher et al., 2008; Khalifeh and Stabel, 2004a,b; Munoz et al., 2008; Weiss et al., 2005). This suggests that a modulation of the immune response is occurring in the clinical animal that surmounts the cell-mediated immunity represented by the presence of IFN-γ in the subclinical animals. In previous studies, the anti-inflammatory cytokines, IL-10 and TGF-β, have demonstrated an ability to facilitate the suppression of mononuclear cell function and IFN-γ secretion (Khalifeh and Stabel, 2004a).

During this ongoing interaction between host immunity and the MAP-infected macrophages, a deviation from the proper immune response against this intracellular pathogen might arise and disrupt the host's attempts to contain the disease (Sohal et al., 2008; Stabel, 2006). This might lead to progressive failure of the macrophages to kill the bacteria, allowing MAP to replicate within the cells and eventually result in death of the macrophages (de Almeida et al., 2008; Sohal et al., 2008; Stabel, 2006). The mechanism(s) by which macrophages fail to contain MAP infection are unclear but NO has been implicated as an effector of macrophage function in other mycobacterial infections (MacMicking et al., 1997). The current study investigated the profile of NO production in unfractionated peripheral blood mononuclear cells (PBMCs) obtained from naturally infected cows in both subclinical and clinical stage of the Johne's disease and noninfected control cows. Exogenous IL-10, TGF-β and IFN-γ were also added to PBMC cultures, in combination with live MAP or LPS, to characterize potential stimulatory or inhibitory roles of these cytokines on NO production by cells from cows in different stages of infection.

2. Materials and methods

2.1. Animals

The animals used in this study were placed in three groups consisting of five noninfected healthy cows, four cows naturally infected with MAP but asymptomatic (i.e., subclinical), and five naturally infected cows with the clinical form of Johne's disease. The stage of infection was determined by fecal shedding of MAP, IFN-γ secretion, and specific antibody response to MAP. Infection was monitored by bacteriologic culture for the fecal shedding of MAP by standard methods (Stabel, 1997). By definition, clinical animals were shedding more than 100 CFU/tube of media (BBL™ Herrold's Egg Yolk Agar Slants with mycobactin J, 2 mg/mL; amphotericin, 50 μg/mL; nalidixic acid, 50 μg/mL; and vancomycin, 50 μg/mL; Becton, Dickinson and Co., Sparks, MD) and presented with weight loss and intermittent diarrhea. Subclinically infected cows were shedding less than 10 CFU/tube and were asymptomatic. The noninfected control cows were characterized by repeated negative fecal cultures performed quarterly over a 3–5-year period and had been purchased from herds with no recent history of Johne's disease. In addition, these animals were negative for production of antibody specific for MAP and IFN-γ performed during that period. All procedures performed on the animals were approved by the Institutional Animal Care and Use Committee (National Animal Disease Center [NADC], Ames, IA).

2.2. Blood collection, culture conditions, and sample collection

Blood was collected from the jugular vein in 2× acid–citrate–dextrose (ACD) for a 1:10 blood dilution. PBMC was isolated from the buffy coat fractions of the peripheral blood and cultured as previously described (Burton and Kehrli, 1996). Briefly, PBMCs were resuspended in RPMI-1640 (Gibco, Grand Island, NY) with 10% fetal calf serum (Atlanta Biologics, Atlanta, GA), 100 U of penicillin G sodium per mL, 100 μg of streptomycin sulfate per mL, 0.25 μg of amphotericin B per mL, and 2 mM L-glutamine (Gibco, Grand Island, NY). Cells were cultured at 2×10^6/mL in 1-mL volumes in 24-well flat-bottomed plates (Nunc, Life Technologies) for 7 days at 39 °C in 5% CO_2 in a humidified atmosphere to allow monocytes in the unfractionated PBMC cultures to develop into macrophages. On day 7, plates were centrifuged at 400 × *g* for 2 min, and the supernatants were removed without disturbing the cells in culture. Cells remaining represented both adherent and non-adherent cell populations as assessed by phenotypic analyses (Khalifeh and Stabel, 2004a). Cells in duplicate wells were then recultured with fresh medium containing 100 ng of bovine IFN-γ (generously donated by Novartis Animal Health, Basel, Switzerland) per mL, 100 ng of human IL-10 (catalog no. 200-10; Peprotech, Rocky Hill, NJ) per mL, and 10 ng of human TGF-β1 (catalog no. 200-21R; Peprotech) per mL or cultured without cytokine stimulation (Khalifeh and Stabel, 2004a). Cultured cells were incubated overnight (18 h), followed by in vitro infection with MAP, strain 19698 (NADC), at a

ratio of 10 bacteria per adherent cell (Stabel, 2000). As a positive-control stimulator of macrophages, replicate wells of in vitro-infected and noninfected cell cultures were also treated with 1 μg of lipopolysaccharide (LPS) (*Escherichia coli* O111:B4-W; Sigma, St. Louis, MO) per mL.

To assess nitric oxide production, cell culture supernatants were collected at 8 h, 3 or 6 days after in vitro infection and stored at −20 °C prior to analysis. The cytokine production as well as MAP survival was previously assessed in these cultures and data were published in a previous study (Khalifeh and Stabel, 2004a). As well, phenotypic analyses were performed 18 h after the replacement of the supernatant in culture with fresh medium and before the addition of MAP to the in vitro cultures (Khalifeh and Stabel, 2004a).

2.3. Bacteria

The MAP strain 19698 (NADC) used in this study was grown in Middlebrook 7H9 broth (pH 6.0) supplemented with mycobactin J (2 mg/L; Allied Monitor, Fayette, MO) and oleic acid–albumin–dextrose complex (Becton Dickinson Microbiology). The bacteria were harvested, washed three times with PBS (pH 7.4), and resuspended to a final concentration of 10^9/mL as determined by the absorbance at 540 nm. Prior to in vitro infection, frozen bacterial stocks were thawed, and clumps were dispersed by brief sonication at 25 W for 40 s with a Tekmar sonic disruptor (Lorton, VA). Previous determinations within the laboratory had demonstrated that brief sonication did not affect the viability of MAP but that storage of frozen stocks at −80 °C might decrease viability by one log. The potential loss of viable bacteria due to freezing was accounted for in the study.

2.4. Assessment of nitric oxide production in the unfractionated PBMC supernatant

To assess nitric oxide production in extended unfractionated PBMC cultures, the stable end product for NO (i.e., NO_3^-, NO_2^-) was analyzed by the Griess reaction (Green et al., 1982; Miranda et al., 2001). Briefly, supernatants were collected from cell cultures at 8 h, 3 and 6 days. Volumes of 100 μL of each sample supernatant, standards (0.1% sodium nitrite) and blank were distributed in 96-well plates. Equal volumes of Griess reaction solutions (0.1% naphthyl-ethylenediamine dihydrochloride and 1% sulfanilamide in 2.5% phosphoric acid) were added to each well. The wells were then incubated in the dark for 10 min at room temperature and the absorbance at 550 nm was measured (PerkinElmer, Gaithersburg, MD).

2.5. Statistical analysis

Data were tested for normality and were found not normally distributed; therefore, non-parametric tests were used. The differences in NO production in the different groups were determined using Wilcoxon sign test. The effect of addition of stimulants and inhibitors was assessed using Kruskal–Wallis test. All statistical analysis was performed using SPSS v 13.

3. Results

3.1. NO production in cows naturally infected with M. avium subsp. paratuberculosis

Macrophages in unfractionated PBMC cultures from subclinically infected cows produced higher ($P < 0.05$) levels of NO at all time points tested compared to the healthy control and clinical Johne's cows (Fig. 1). Effects due to time were not noted in cell cultures from subclinically infected cows, whereas the level of NO production increased ($P < 0.05$) between 8 h and 6 days of culture (Fig. 1) in clinical cows. It was also demonstrated that clinical Johne's cows had lower ($P < 0.05$) NO production than the healthy animals at the early time points tested (8 h and 3 days) (Fig. 1). By 6 days, the level of NO production for clinical cows was similar to that detected in healthy cows.

3.2. Upregulation of NO production by MAP and rIFN-γ

Infection of macrophages in unfractionated PBMC obtained from healthy animals with live MAP resulted in increased ($P < 0.05$) levels of NO production compared to noninfected cell cultures at two time points (3 and 6 days) (Fig. 2B and C). Similar results were also observed for clinical cows with significant response at 6 days (Fig. 2C). The addition of live MAP to cell cultures from subclinical animals did not result in further increases in NO production at any of the time points (Fig. 2).

Additive effects of rIFN-γ were noted when it was added to cultures prior to MAP infection. Co-stimulation of cell cultures with rIFN-γ and live MAP resulted in an increase ($P < 0.05$) in the level of NO production with time in all groups (3 and 6 days) (Fig. 2). An increase ($P < 0.05$) in NO production was noted at 3 and 6 days post-infection in naturally infected cows but effects were most dramatic

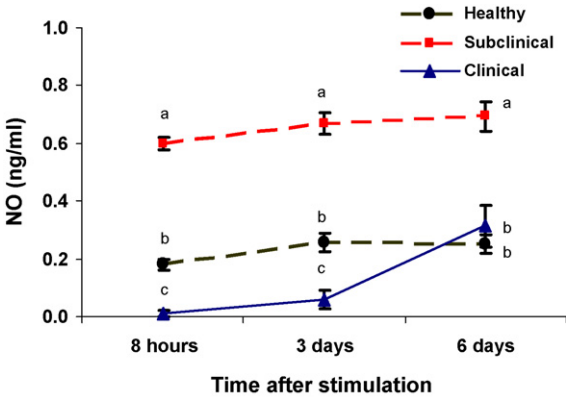

Fig. 1. The profile of nitric oxide production by unfractionated PBMC in cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) at subclinical and clinical stages of the disease compared to noninfected healthy animals. PBMC culture medium was replaced with complete medium on day 6. Cell culture supernatants were collected 8 h, 3 and 6 days after media change. The nitric oxide produced in the supernatants was measured and represented as means ± SEM. Letters indicate statistically significant differences between treatment groups within each time point ($P < 0.05$).

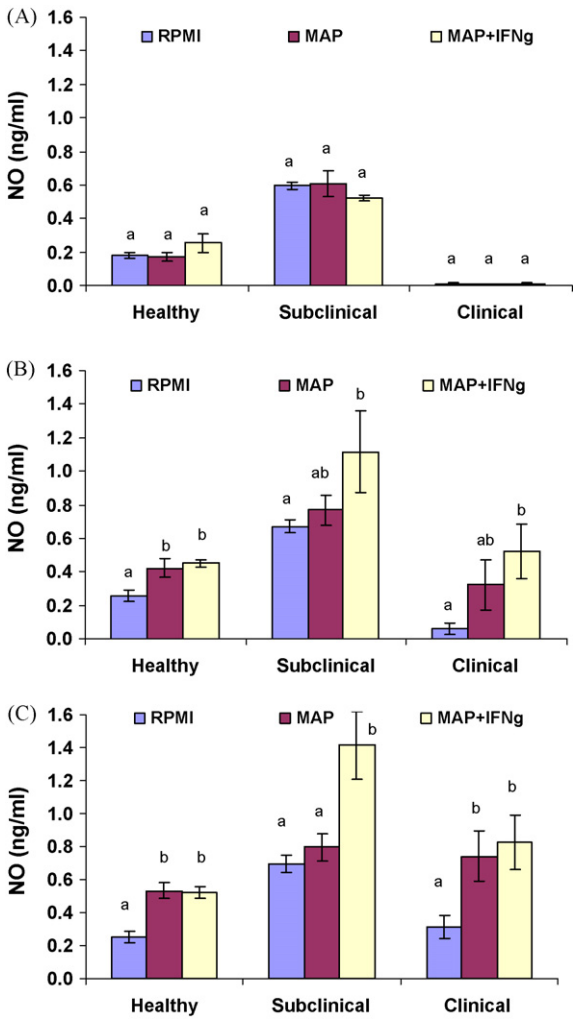

Fig. 2. Nitric oxide production in the unfractionated PBMC cultures of noninfected healthy cows and cows with subclinical and clinical Johne's disease upon addition of live *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and IFN-γ. PBMC culture medium was replaced on day 6 with complete medium containing rIFN-γ (100 ng/mL). After 18 h (day 7), cells in culture were infected with live MAP (10:1 bacterium-to-adherent cell ratio). Culture supernatants were collected at 8 h (A), 3 days (B), and 6 days (C) after bacterial stimulation. The nitric oxide produced in the supernatants was measured and represented as means ± SEM. Letters indicate statistically significant differences between treatment groups within each time point ($P < 0.05$).

($P < 0.05$) in the subclinically infected cows. Interestingly, NO production was not altered by the addition of rIFN-γ alone (100 ng/mL) to cell cultures, regardless of the infection status of the cows (data not shown). It is also important to mention that the NO production in cell cultures containing rIFN-γ and infected with MAP for 8 h showed no significant differences among groups (Fig. 2A).

3.3. NO production after nonspecific stimulation with E. coli O111:B4-W LPS and rIFN-γ

NO production was significantly ($P < 0.05$) higher for PBMC isolated from subclinically infected cows after 8 h of culture, with no added effects observed after in vitro stimulation with LPS or LPS+ rIFN-γ in any infection group (Fig. 3A). At 3 days after LPS stimulation, an increase in NO production was observed in all animal groups while cell cultures from subclinical cows still had higher levels of NO production compared to the other groups (Fig. 3B). It was noted that when macrophages in the unfractionated PBMC cultures were stimulated with *E. coli* O111:B4-W LPS for 6 days, cells from the clinical cows produced similar levels of NO to that measured in the culture obtained from the subclinical cows at the same time point (Fig. 3C). Overall, moderate increases in NO production were demonstrated between 8 h and 3 days after in vitro stimulation with *E. coli* O111:B4-W LPS for cells from healthy and subclinically infected cows but a more protracted and highly significant

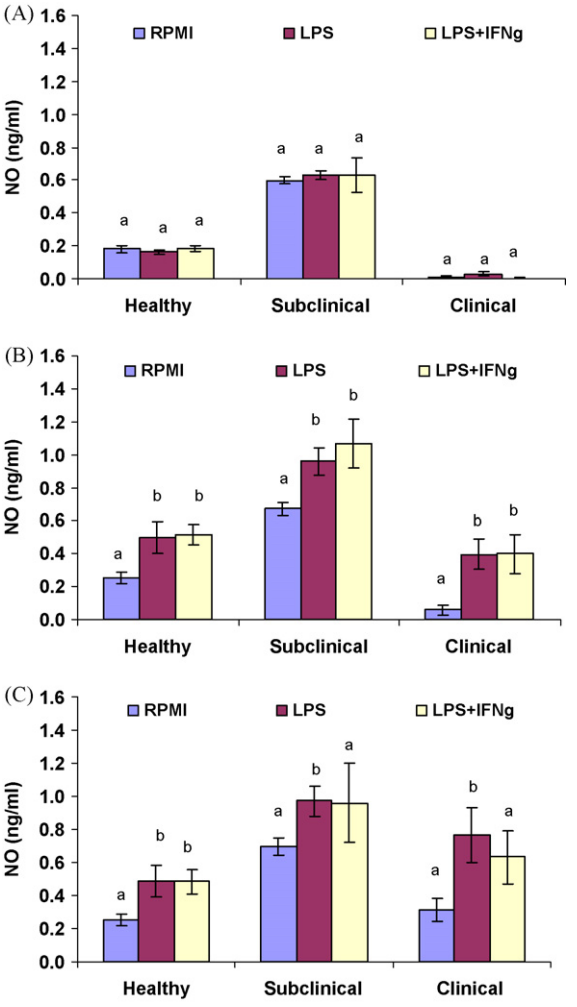

Fig. 3. Nitric oxide production in the unfractionated PBMC cultures of noninfected healthy cows and cows with subclinical and clinical Johne's disease upon addition of LPS (*E. coli* O111:B4) and IFN-γ. PBMC culture medium was replaced on day 6 with complete medium containing recombinant IFN-γ (100 ng/mL). After 18 h (day 7), cells in culture were stimulated with LPS (1 μg/mL). Culture supernatants were collected at 8 h (A), 3 days (B), and 6 days (C) after LPS stimulation. The nitric oxide produced in the supernatants was measured and represented as means ± SEM. Letters indicate statistically significant differences between treatment groups within each time point ($P < 0.05$).

increase ($P < 0.05$) in the level of NO production was noted over time for clinically infected cows (8 h to 6 days) (Fig. 3).

3.4. Downregulation of NO production by IL-10 and TGF-β

The addition of both IL-10 and TGF-β to PBMC cultures prior to infection with live MAP resulted in a decrease ($P < 0.05$) in NO production in the healthy and subclinical groups regardless of the period of incubation after MAP in vitro infection (8 h, 3 or 6 days) (Fig. 4). Similar results were noted when the PBMCs were stimulated with *E. coli* O111:B4-W LPS instead of MAP (Fig. 5). In clinical cows, a decrease in NO production was not apparent until day 6 after the in vitro infection with MAP (Fig. 4C) while the

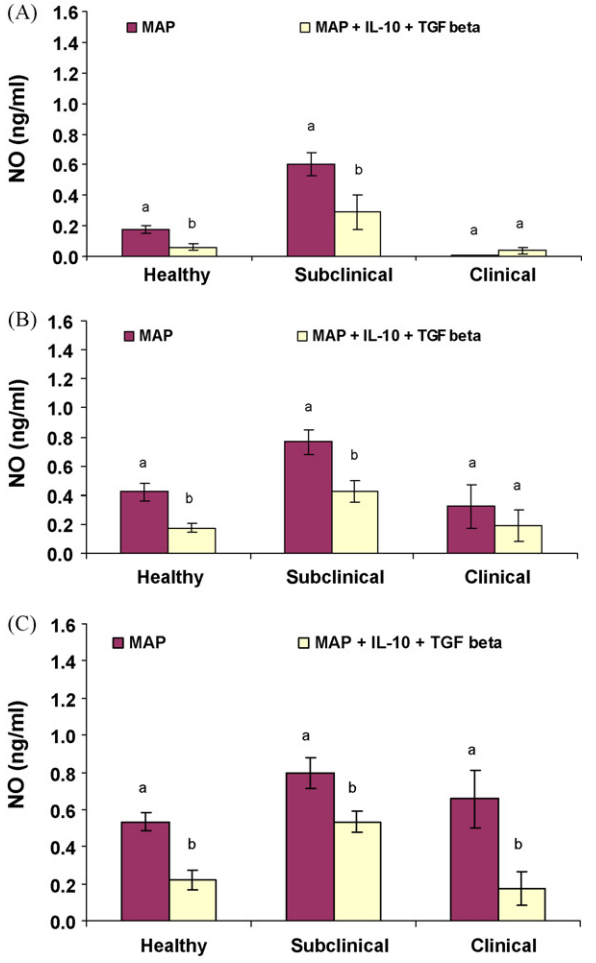

Fig. 4. Nitric oxide production in the unfractionated PBMC cultures of noninfected healthy cows and cows with subclinical and clinical Johne's disease upon addition of live *Mycobacterium avium* subsp. *paratuberculosis* and rIL-10 plus rTGF-β. PBMC culture medium was replaced on day 6 with complete medium containing rIL-10 (100 ng/mL) plus rTGF-β (10 ng/mL). After 18 h (day 7), cells in culture were infected with live MAP (10:1 bacterium-to-adherent cell ratio). Culture supernatants were collected at 8 h (A), 3 days (B), and 6 days (C) after bacterial infection. The nitric oxide produced in the supernatants was measured and represented as means ± SEM. Letters indicate statistically significant differences between treatment groups within each time point ($P < 0.05$).

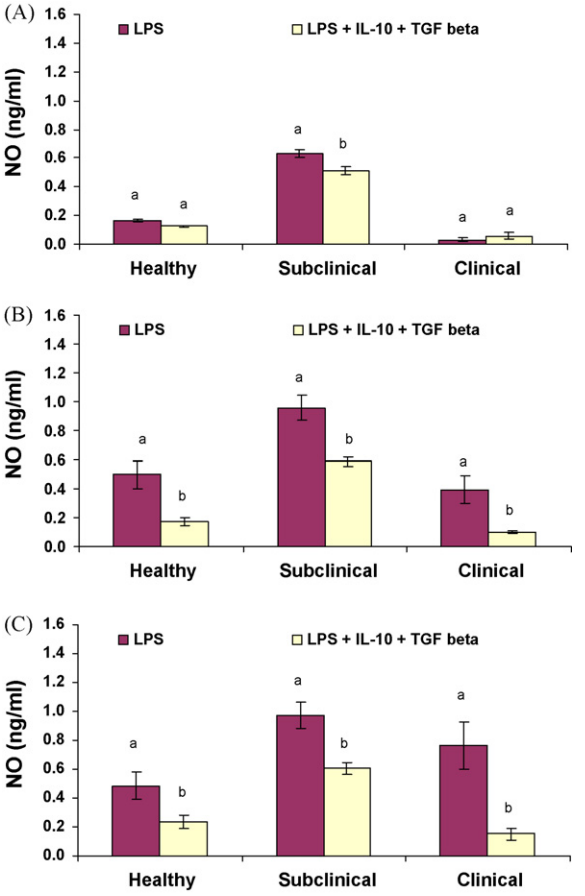

Fig. 5. Nitric oxide production in the unfractionated PBMC cultures of noninfected healthy cows and cows with subclinical and clinical Johne's disease upon addition of LPS (*E. coli* O111:B4) and rIL-10 plus rTGF-β. PBMC culture medium was replaced on day 6 with complete medium containing rIL-10 (100 ng/mL) plus rTGF-β (10 ng/mL). After 18 h (day 7), cells in culture were stimulated with LPS (1 μg/mL). Culture supernatants were collected at 8 h (A), 3 days (B), and 6 days (C) after LPS stimulation. The nitric oxide produced in the supernatants was measured and represented as means ± SEM. Letters indicate statistically significant differences between treatment groups within each time point ($P < 0.05$).

inhibitory effects of added IL-10 and TGF-β were observed earlier (day 3) after O111:B4-W LPS stimulation (Fig. 5B and C). In the absence of *E. coli* O111:B4-W LPS stimulation or MAP in vitro infection, only cell cultures from subclinically infected cows demonstrated decreased NO production in response to the addition of IL-10 and TGF-β and the inhibition was only apparent in cell cultures after 6 days (data not shown).

4. Discussion

This study contributes to the unraveling of protective mechanisms involved in cattle initially controlling MAP infection before subsequently evolving to the clinical stage of disease. It describes the influence of animal infection status as well as a variety of culture conditions (exogenous cytokines, culture period, nonspecific and specific stimulators) on the release of NO by PBMCs. Interestingly, macrophage function assays were carried out up to 6 days

post-antigenic stimulation, in contrast to other studies based upon 20–96 h assays, providing a different insight into immune responsiveness of animals in different stages of infection (Liebana et al., 2000; Weiss et al., 2005). Cells displayed different phenotypes due to the differences in natural infection status but these differences were not affected by the protracted incubation period (Khalifeh and Stabel, 2004a; Stabel and Khalifeh, 2008; Weiss et al., 2005; Zhao et al., 1997). Temporal studies of this type are useful in providing information on the effects of cumulative levels of cytokine in culture and the dynamic between cell types as influenced by these cytokines and host infection status.

The role of NO in the control of MAP infection is not well established, and to the best of our knowledge, production of this mediator has been measured only in cultures from which cells were obtained from healthy animals and then stimulated in vitro with MAP. The present data demonstrated that animals naturally infected with MAP and in the subclinical stage of the disease had the highest level of NO production among groups. In contrast, NO production was the lowest in the clinically infected animals, specifically at 8 h and 3 days post-infection. Cows with clinical Johne's disease have a strong Th2-mediated humoral immune response associated with anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β (Khalifeh and Stabel, 2004b; Munoz et al., 2008; Oswald et al., 1992; Ren et al., 2008). The inhibitory effect of these anti-inflammatory cytokines on macrophage iNOS generation has been demonstrated in other model systems (Goff et al., 1998a; Mitani et al., 2005). In the mouse model, iNOS inhibition by Th2 cytokines is mediated by the induction of the arginase pathway that leads to the generation of L-ornithine and urea instead of nitric oxide (Munder et al., 1998, 1999). The arginase pathway is inhibited by Th1 cytokines, including IFN-γ, a strong inducer of iNOS generation in activated macrophages. Therefore, the profile of NO production reported in this study agrees with the distinct patterns of cytokine expression previously reported for subclinically and clinically infected cows (Khalifeh and Stabel, 2004a; Munoz et al., 2008; Weiss et al., 2005).

The majority of previously published studies have correlated NO production with its effect on mycobacterial survival within the macrophage (Axelrod et al., 2008; Zhao et al., 1997). However, it is also clear that no singular mechanism or cell type is sufficient to kill mycobacteria in vivo (Zhao et al., 1997). Therefore, defining the role of NO in host defense without regard for other effectors is difficult. Previously, we reported that the killing ability of monocyte-derived macrophages within the unfractionated cell cultures that were obtained from either subclinical or clinical animals did not differ (Khalifeh and Stabel, 2004a). Given this information, it seems likely that regardless of the difference that was obtained among groups for NO production, these differences do not reflect upon the killing ability of cells in response to in vitro infection with MAP (Khalifeh and Stabel, 2004a).

Alternatively, correlating the cytokine profile presented within the cell cultures with the impairment of NO responsiveness may provide some understanding of the effects of disease state on host immune function. Previously, it was shown that in vitro infection of unfractionated PBMC cultures from healthy noninfected animals with live MAP resulted in an increase in IFN-γ production, an effect that was correlated with a lower intracellular bacterial burden (Khalifeh and Stabel, 2004a). This profile is also in agreement with data that showed that stimulation of monocyte-derived macrophages with IFN-γ resulted in a decrease in MAP survival and that mycobactericidal activity was accompanied by an increase in NO production (Zhao et al., 1997). In contrast, NO production by bovine macrophages increased after 24 h of incubation with MAP, with and without IFN-γ, and this increase could not be directly linked to increased bacterial killing (Weiss et al., 2002).

Cows with subclinical Johne's disease generally have high IFN-γ responses, an indicator of strong cell-mediated immunity (Khalifeh and Stabel, 2004b; Sohal et al., 2008; Stabel, 2000) and low anti-inflammatory cytokine production (de Almeida et al., 2008; Karcher et al., 2008; Khalifeh and Stabel, 2004a,b). In the present study, subclinically infected cows had higher baseline levels of NO production compared to healthy controls and clinical cows and NO levels increased further upon incubation of cells with MAP and IFN-γ but not with MAP alone. These observations suggest that MAP infection will induce a NO response by naïve macrophages obtained from noninfected animals but the presence of IFN-γ may be required for the induction of responses to MAP infection in cells from subclinically infected animals that have high innate NO production.

The survival of MAP within the animal or in culture after NO upregulation may be due to the ability of mycobacteria to evolve strategies to combat the toxic effects of NO and cause an impairment of NO responsiveness. For example, *M. tuberculosis* ensures its survival within macrophages through the inhibition of iNOS recruitment to its phagosome through decreased expression of EBP50, a scaffolding protein required for mycobacterial viability (Davis et al., 2007). Stimulation with IFN-γ and LPS resulted in an increase in EBP50 expression, perhaps through an autocrine augmentation of IFN-γ production (Davis et al., 2007). This can be extrapolated to the current study where the addition of IFN-γ to cell cultures prior to MAP infection resulted in an increase in NO production. Therefore, in the continuous presence of MAP along with high levels of IFN-γ and low levels of anti-inflammatory cytokines, NO production may be enough to control but not eliminate the bacterial burden.

Singular effects of MAP infection were noted in unfractionated cell cultures obtained from clinical animals, resulting in an increased NO production. In a previous study, higher levels of IFN-γ were reported upon the addition of live MAP to cell cultures from clinically infected cows as compared to uninfected cultures (Khalifeh and Stabel, 2004a). In addition, in vitro infection of cell cultures from both subclinical and clinical animals with live MAP resulted in the upregulation of anti-inflammatory cytokines, IL-10 and TGF-β (Khalifeh and Stabel, 2004a). This suggests a potential downregulatory effect of IL-10 and TGF-β on NO production and cell responsiveness even in the presence of high levels of IFN-γ This is confirmed by the reduction in NO production observed in all experimental groups upon

the addition of IL-10 and TGF-β to cell cultures prior to MAP infection. Further, it has been demonstrated that endogenous or exogenous IL-10 results in the downregulation of iNOS expression in cultured bovine monocyte-derived macrophages (Goff et al., 1998b). Neutralization of IL-10 doubled the ability of infected macrophages to kill MAP and increased production of NO (Weiss et al., 2005). This downregulatory activity of IL-10 is prominent even during sustained IFN-γ release by γδ T cells in the peripheral blood (Goff et al., 1998b). Similarly, the endogenous TGF-β produced during the activation of murine peritoneal macrophages suppressed the induction of iNOS (Jun et al., 1995). The absence of TGF-β with consequent rIFN-γ stimulation resulted in more NO production compared with cells that were stimulated with rIFN-γ without TGF-β inhibition (Jun et al., 1995).

Nonspecific stimulation of cells with LPS (*E. coli* O111:B4) resulted in similar increases in NO production for healthy controls and clinical cows to that noted after the addition of live MAP. LPS also increased NO production in cell cultures from subclinically infected cows, an effect that is in contrast to results obtained after MAP infection. Nitric oxide production was not augmented further upon the addition of both LPS and IFN-γ to cell cultures, regardless of treatment group. These results suggest that cows in the subclinical stage of infection may display a state of cellular unresponsiveness that is specifically associated with live MAP. To further support this theory, it has been shown that EBP50 expression is downregulated after the addition of live *M. tuberculosis* to macrophages whereas dead *M. tuberculosis* or LPS did not show the same downregulatory effect (Davis et al., 2007).

5. Conclusions

In the present study subclinically infected cows had higher NO production compared to healthy controls or clinically infected cows, demonstrating that disease state can have a profound effect on the release of NO from macrophages. In addition, cells from subclinically infected cows did not respond to infection with live MAP but the co-stimulation of cells with MAP and IFN-γ resulted in the highest level of NO production. Hypothetically, the transition of the infection status in cattle from subclinical to clinical disease may be a result of an increase in the bacterial load of the cells, ceasing further IFN-γ upregulation and leading to impairment in the responsiveness to the intracellular killing mechanisms such as NO generation.

References

Adams, L.B., Franzblau, S.G., Vavrin, Z., Hibbs Jr., J.B., Krahenbuhl, J.L., 1991. L arginine-dependent macrophage effector functions inhibit metabolic activity of *Mycobacterium leprae*. J. Immunol. 147, 1642–1646.

Axelrod, S., Oschkinat, H., Enders, J., Schlegel, B., Brinkmann, V., Kaufmann, S.H., Haas, A., Schaible, U.E., 2008. Delay of phagosome maturation by a mycobacterial lipid is reversed by nitric oxide. Cell. Microbiol. 10, 1530–1545.

Burton, J.L., Kehrli Jr., M.E., 1996. Effects of dexamethasone on bovine circulating T lymphocyte populations. J. Leukoc. Biol. 59, 90–99.

Chan, J., Xing, Y., Magliozzo, R.S., Bloom, B.R., 1992. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. J. Exp. Med. 175, 1111–1122.

Davis, A.S., Vergne, I., Master, S.S., Kyei, G.B., Chua, J., Deretic, V., 2007. Mechanism of inducible nitric oxide synthase exclusion from mycobacterial phagosomes. PLoS Pathog. 3, e186.

de Almeida, D.E., Colvin, C.J., Coussens, P.M., 2008. Antigen-specific regulatory T cells in bovine paratuberculosis. Vet. Immunol. Immunopathol. 125, 234–245.

Denis, M., 1991. Interferon-gamma-treated murine macrophages inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates. Cell. Immunol. 132, 150–157.

Goff, W.L., Johnson, W.C., Cluff, C.W., 1998a. Babesia bovis immunity. In vitro and in vivo evidence for IL-10 regulation of IFN-gamma and iNOS. Ann. N Y Acad. Sci. 849, 161–180.

Goff, W.L., O'Rourke, K.I., Johnson, W.C., Lacy, P.A., Davis, W.C., Wyatt, C.R., 1998b. The role of IL-10 in iNOS and cytokine mRNA expression during in vitro differentiation of bovine mononuclear phagocytes. J. Interferon Cytokine Res. 18, 139–149.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal. Biochem. 126, 131–138.

Hostetter, J.M., Steadham, E.M., Haynes, J.S., Bailey, T.B., Cheville, N.F., 2002. Cytokine effects on maturation of the phagosomes containing *Mycobacterium avium* subspecies *paratuberculosis* in J774 cells. FEMS Immunol. Med. Microbiol. 34, 127–134.

Jun, C.D., Choi, B.M., Kim, S.U., Lee, S.Y., Kim, H.M., Chung, H.T., 1995. Down regulation of transforming growth factor-beta gene expression by antisense oligodeoxynucleotides increases recombinant interferon-gamma-induced nitric oxide synthesis in murine peritoneal macrophages. Immunology 85, 114–119.

Karcher, E.L., Beitz, D.C., Stabel, J.R., 2008. Modulation of cytokine gene expression and secretion during the periparturient period in dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*. Vet. Immunol. Immunopathol. 123, 277–288.

Khalifeh, M.S., Stabel, J.R., 2004a. Effects of gamma interferon, interleukin-10, and transforming growth factor beta on the survival of *Mycobacterium avium* subsp. *paratuberculosis* in monocyte-derived macrophages from naturally infected cattle. Infect. Immun. 72, 1974–1982.

Khalifeh, M.S., Stabel, J.R., 2004b. Upregulation of transforming growth factor-beta and interleukin-10 in cows with clinical Johne's disease. Vet. Immunol. Immunopathol. 99, 39–46.

Lee, H., Stabel, J.R., Kehrli Jr., M.E., 2001. Cytokine gene expression in ileal tissues of cattle infected with *Mycobacterium paratuberculosis*. Vet. Immunol. Immunopathol. 82, 73–85.

Liebana, E., Aranaz, A., Welsh, M., Neill, S.D., Pollock, J.M., 2000. In vitro T-cell activation of monocyte-derived macrophages by soluble messengers or cell-to-cell contact in bovine tuberculosis. Immunology 100, 194–202.

MacLean, A., Wei, X.Q., Huang, F.P., Al-Alem, U.A., Chan, W.L., Liew, F.Y., 1998. Mice lacking inducible nitric-oxide synthase are more susceptible to herpes simplex virus infection despite enhanced Th1 cell responses. J. Gen. Virol. 79 (Pt 4), 825–830.

MacMicking, J., Xie, Q.W., Nathan, C., 1997. Nitric oxide and macrophage function. Ann. Rev. Immunol. 15, 323–350.

Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 5, 62–71.

Mitani, T., Terashima, M., Yoshimura, H., Nariai, Y., Tanigawa, Y., 2005. TGF-beta1 enhances degradation of IFN-gamma-induced iNOS protein via proteasomes in RAW 264 7 cells. Nitric Oxide 13, 78–87.

Munder, M., Eichmann, K., Modolell, M., 1998. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4+ T cells correlates with Th1/Th2 phenotype. J. Immunol. 160, 5347–5354.

Munder, M., Eichmann, K., Moran, J.M., Centeno, F., Soler, G., Modolell, M., 1999. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. J. Immunol. 163, 3771–3777.

Munoz, M., Delgado, L., Verna, A., Benavides, J., Garcia-Pariente, C., Fuertes, M., Ferreras, M.C., Garcia-Marin, J.F., Perez, V., 2008. Expression of transforming growth factor-beta 1 (TGF-beta1) in different types of granulomatous lesions in bovine and ovine paratuberculosis. Comp. Immunol. Microbiol. Infect. Dis. (Epub ahead of print).

Oswald, I.P., Gazzinelli, R.T., Sher, A., James, S.L., 1992. IL-10 synergizes with IL-4 and transforming growth factor-beta to inhibit macrophage cytotoxic activity. J. Immunol. 148, 3578–3582.

Ren, Z., Turton, J., Borody, T., Pang, G., Clancy, R., 2008. Selective Th2 pattern of cytokine secretion in *Mycobacterium avium* subsp. *paratuberculosis* infected Crohn's disease. J. Gastroenterol. Hepatol. 23, 310–314.

Sohal, J.S., Singh, S.V., Tyagi, P., Subhodh, S., Singh, P.K., Singh, A.V., Narayanasamy, K., Sheoran, N., Singh Sandhu, K., 2008. Immunology

of mycobacterial infections: with special reference to *Mycobacterium avium* subspecies *paratuberculosis*. Immunobiology 213, 585–598.

Stabel, J.R., 1997. An improved method for cultivation of *Mycobacterium paratuberculosis* from bovine fecal samples and comparison to three other methods. J. Vet. Diagn. Invest. 9, 375–380.

Stabel, J.R., 2000. Cytokine secretion by peripheral blood mononuclear cells from cows infected with *Mycobacterium paratuberculosis*. Am. J. Vet. Res. 61, 754–760.

Stabel, J.R., 2006. Host responses to *Mycobacterium avium* subsp. *paratuberculosis*: a complex arsenal. Anim. Health Res. Rev. 7, 61–70.

Stabel, J.R., Khalifeh, M.S., 2008. Differential expression of CD5 on B lymphocytes in cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*. Vet. Immunol. Immunopathol. 126, 211–219.

Sweeney, R.W., Jones, D.E., Habecker, P., Scott, P., 1998. Interferon-gamma and interleukin 4 gene expression in cows infected with *Mycobacterium paratuberculosis*. Am. J. Vet. Res. 59, 842–847.

Taylor-Robinson, A.W., Liew, F.Y., Severn, A., Xu, D., McSorley, S.J., Garside, P., Padron, J., Phillips, R.S., 1994. Regulation of the immune response by nitric oxide differentially produced by T helper type 1 and T helper type 2 cells. Eur. J. Immunol. 24, 980–984.

Weiss, D.J., Evanson, O.A., Moritz, A., Deng, M.Q., Abrahamsen, M.S., 2002. Differential responses of bovine macrophages to *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium*. Infect. Immun. 70, 5556–5561.

Weiss, D.J., Evanson, O.A., de Souza, C., Abrahamsen, M.S., 2005. A critical role of interleukin-10 in the response of bovine macrophages to infection by *Mycobacterium avium* subsp *paratuberculosis*. Am. J. Vet. Res. 66, 721–726.

Zhao, B., Collins, M.T., Czuprynski, C.J., 1997. Effects of gamma interferon and nitric oxide on the interaction of *Mycobacterium avium* subsp. *paratuberculosis* with bovine monocytes. Infect. Immun. 65, 1761–1766.